UNIVERSIDADE FEDERAL DO PARANÁ

CAROLINE NOGOZZEKI CAMARGO

EXPRESSÃO GÊNICA NO MODELO ANIMAL DA DOENÇA DE ALZHEIMER INDUZIDA PELA ESTREPTOZOTOCINA

CURITIBA

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EXPRESSÃO GÊNICA NO MODELO ANIMAL DA DOENÇA DE ALZHEIMER INDUZIDA PELA ESTREPTOZOTOCINA

Dissertação apresentada como requisito parcial à obtenção do grau de Mestre em Farmacologia, no Curso de Pós-Graduação em Farmacologia, Setor de Ciências Biológicas, Universidade Federal do Paraná.

Orientadora: Prof^a Dr^a Maria A. B. F. Vital
Co-orientadora: Dr^a Taysa Bervian Bassani

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Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em FARMACOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da dissertação de Mestrado de **CAROLINE NOGOZZEKI CAMARGO** intitulada: **Expressão gênica no modelo animal da doença de Alzheimer induzida pela estreptozotocina**, após terem inquirido a aluna e realizado a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

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CURITIBA, 17 de Abril de 2019.

MARIA APARECIDA BARBATO FRAZÃO VITAL
Presidente da Banca Examinadora (UFPR)

Adriana F. Mercadante
ADRIANA FROHLICH MERCADANTE
Avaliador Externo (UFPR)

ROBERTO ANDREATINI
Avaliador Interno (UFPR)

Ao meu marido Robinson de Souza Leão, ao meu filho Nicolas Nogozzeki Leão e à minha mãe Maria Carmelita Nogozzeki (in memorian).

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RESUMO

A doença de Alzheimer (DA) é a forma mais frequente de demência, é o distúrbio neurodegenerativo mais prevalente no mundo, pode ser caracterizada clinicamente por um progressivo comprometimento da função cognitiva durante a vida média e tardia do adulto, sendo os sintomas iniciais tipicamente certas formas de perda de memória e de linguagem. O modelo animal gerado pela administração intracerebroventricular (ICV) de estreptozotocina (STZ), mostra muitos aspectos da DA esporádica. A neuroinflamação, manifestada pela ativação microglial, é um componente importante da patologia da doença de Alzheimer, elevações em marcadores neuroinflamatórios são amplamente relatadas na DA, tanto em pacientes humanos quanto em modelos animais. O objetivo deste estudo foi investigar alterações na expressão dos genes Allograft Inflammatory Factor-1 (*AIF1*), também conhecido como gene que codifica a proteína adaptadora de ligação ionizada de cálcio (Iba1) e tem sua expressão aumentada no processo inflamatório e glicogênio sintase quinase-3β (*GSK3B*) que é a principal quinase que fosforila tau e uma das quinases sugeridas estar envolvida na sua hiperfosforilação aberrante na DA. Os animais foram injetados com STZ-ICV ou veículo e avaliados em testes comportamentais no período de quatro semanas, no dia 27 no Teste de Reconhecimento de Objetos (TRO), no dia 28 na versão espacial do labirinto em Y e teste de campo aberto. Foram pesados no início do experimento e a cada 7 dias após a cirurgia, até o 28º dia, no dia 30 foram eutanasiados e os cérebros foram dissecados para quantificação da expressão gênica do gene *AIF1* e *GSK3B* no hipocampo e no córtex pré-frontal. Trinta dias após a cirurgia, animais infundidos com STZ exibiram prejuízo na memória espacial de curto prazo (labirinto em Y) e memória de reconhecimento de curto prazo no teste de reconhecimento de objetos. A expressão do gene *AIF1*, mas não *GSK3B*, foi significativamente elevada apenas no hipocampo do grupo STZ-ICV em comparação com o grupo Sham. Esses dados sugerem uma participação da neuroinflamação nesse modelo animal de DA esporádica.

Palavras-chave: Doença de Alzheimer. Estreptozotocina. *AIF1*. *GSK3B*. Neuroinflamação. Memória.

ABSTRACT

Alzheimer's disease (AD) is the most frequent form of dementia, it is the most prevalent neurodegenerative disorder in the world, it can be characterized clinically by a progressive impairment of cognitive function during the average and late life of the adult, the initial symptoms typically being certain forms of memory loss and language. The animal model generated by the intracerebroventricular (ICV) administration of streptozotocin (STZ), shows many aspects of sporadic AD. Neuroinflammation, manifested by microglial activation, is an important component of the pathology of Alzheimer's disease, elevations in neuroinflammatory markers are widely reported in AD in both human and animal models. The aim of this study was to investigate alterations in the expression of Allograft Inflammatory Factor-1 (*AIF1*) genes, also known as the gene that encodes the ionized calcium binding adapter protein (Iba1) and has increased expression in the inflammatory process and glycogen synthase kinase- 3β (*GSK3B*) which is the main kinase phosphorylating tau and one of the suggested kinases is involved in its aberrant hyperphosphorylation in AD. The animals were injected with STZ-ICV or vehicle and evaluated in behavioral tests in the period of four weeks, day 27 in the Object Recognition Test (ORT), day 28 in the spatial version of the Y-maze and open field test (OFT). They were weighed at the beginning of the experiment and every 7 days after surgery until the 28th day, on day 30 they were euthanized and the brains were dissected for quantification of the gene expression of the *AIF1* and *GSK3B* gene in the hippocampus and the prefrontal cortex. Thirty days after surgery, animals infused with STZ exhibited impairment in short-term spatial memory (Y-labyrinth) and short-term recognition memory in the object recognition test (ORT). Expression of the AIF1 gene, but not GSK3B, was significantly elevated only in the hippocampus of the STZ-ICV group compared to the Sham group. These data suggest a role of neuroinflammation in this animal model of sporadic AD.

Key-words: Alzheimer’s disease. Streptozotocin. *AIF1*. *GSK3B*.

Neuroinflammation. Memory.

SUMÁRIO

1 INTRODUÇÃO À DOENÇA DE ALZHEIMER

A doença de Alzheimer (DA) é a forma mais frequente de demência, é uma desordem neurodegenerativa progressiva, contínua, que afeta amplas áreas do córtex cerebral e hipocampo ((BLENNOW *et al*., 2006; MASTERS et al., 2015), é o distúrbio neurodegenerativo mais prevalente no mundo, afetando cerca de 24 milhões de pessoas e estima-se que até 2050 este número será quadruplicado, estimativas em 2015 mostraram que cerca de 46,8 milhões de pessoas são afetadas por demência em todo o mundo. Esse número de casos novos é quase 30% (9,9 milhões de novos casos) maior que a incidência apresentada no relatório da Organização Mundial da Saúde (OMS) em 2010 (PICANÇO et al., 2018).

A DA pode ser caracterizada clinicamente por um progressivo comprometimento da função cognitiva durante a vida média e tardia do adulto, sendo os sintomas iniciais tipicamente certas formas de perda de memória e de linguagem (AULD et al., 2002). Em conjunto, o manejo não farmacológico e farmacológico na DA procura minimizar os efeitos incapacitantes do declínio cognitivo e funcional e a emergência e gravidade dos sintomas comportamentais e psicológicos da demência (ATRI, 2019). Os tratamentos sintomáticos para a doença de Alzheimer estão amplamente disponíveis desde meados da década de 1990 (BALLARD et al., 2011). Os medicamentos para DA aprovados pela FDA, inibidores da colinesterase, donepezila, a galantamina e a rivastigmina, e antagonista do N-metil-d-aspartato (NMDA) a memantina, podem reduzir a progressão dos sintomas clínicos e da incapacidade (ATRI, 2019).

1.1 ETIOLOGIA E PATOGÊNESE DA DOENÇA DE ALZHEIMER

Dependendo da idade de início, a DA manifesta-se como: DA de início precoce ou familiar e de início tardio ou esporádica. DA familiar é geralmente associada a mutações nos genes da presenilina 1 (PSEN1) e presenilina 2 (PSEN2) e da proteína precursora amiloide (APP), os sintomas se desenvolvem mais precocemente do que na forma esporádica, tipicamente em pacientes com menos de 65 anos de idade (DUBEY et al., 2019; LANE et al., 2017), enquanto a DA esporádica, é principalmente associada ao constante enfraquecimento e dano das células nervosas e função cerebral com o envelhecimento (DUBEY et al., 2019). A maioria dos casos de DA são esporádicos e resultam de múltiplos fatores etiológicos, incluindo fatores ambientais, genéticos e metabólicos (MASTERS *et al*., 2015). Estudos de associação genômica, utlizando uma ampla gama de amostras identificaram mais de 20 fatores de risco genéticos, dentre eles o metabolismo inflamatório e do colesterol (LANE et al., 2017).

Em particular, a ativação da micróglia em resposta à deposição amilóide é agora reconhecida como tendo um papel chave na patogênese da DA. Esses genes de risco relativamente comuns conferem apenas um risco aumentado muito pequeno, mas quando combinados em um escore de risco poligênico, podem quase dobrar a chance de ocorrer (LANE et al., 2017).

Apesar do fato de que muitos avanços na patogênese e na prática clínica foram alcançados nas últimas décadas, os fatores que desencadeiam o início e a progressão da DA permanecem pouco esclarecidos (SUN et al., 2018.)

A doença de Alzheimer está associada ao acúmulo de proteína β-amilóide (Aβ) insolúvel em placas no espaço extracelular, assim como na parede dos vasos sanguíneos, e agregação da proteína tau dos microtúbulos em emaranhados neurofibrilares no ambiente intracelular dos neurônios, sendo essas as principais características da patologia da doença (MASTERS et al., 2015; LANE et al., 2017), essas alterações causam morte neuronal e perda de sinapses, todas contribuindo para o declínio cognitivo progressivo (KOCAHAN, et al., 2017).

A hipótese amilóide, a teoria prevalente da patogênese da DA, sugere que o acúmulo de formas patológicas de Aβ produzidas pela clivagem seqüencial da APP pelas enzimas β- e γ-secretase no cérebro é o processo patológico primário, conduzido por um desequilíbrio entre produção Aβ e remoção Aβ. A formação de agregados neurofibrilares e subsequente disfunção neuronal e neurodegeneração, talvez mediada via inflamação, são consideradas processos posteriores (LANE et al., 2017).

1.2 MODELO ANIMAL

O modelo animal gerado pela administração intracerebroventricular (icv) de estreptozocina (STZ), mostra muitos aspectos da DA esporádica (CHEN *et al*., 2013) como déficits cognitivos e colinérgicos do cérebro, stress oxidativo bem como uma diminuição na glicose cerebral/ metabolismo energético e estado cerebral resistente à insulina (SALKOVIC-PETRISIC *et al*., 2012).

Quimicamente, a estreptozotocina (STZ) é uma glicosamina – nitrosoureia (More *et al*., 2016), isolado no final da década de 1950 a partir de uma cepa da bactéria do solo Streptomyces achromogenes. Foi patenteada e inicialmente desenvolvido como antibiótico, posteriormente como agente anticancerígeno (GRIEB, 2015) e tem sido extensivamente investigada na pesquisa pré-clínica, porque injetada intravenosamente ou intraperitonealmente induz o diabetes em animais experimentais (GRIEB, 2015; More *et al*., 2016). O cérebro não é afetado diretamente porque a STZ não é capaz de penetrar na barreira hematoencefálica que não possui o transportador GLUT2 (GRIEB, 2015). O modelo animal de STZ icv foi introduzido por Lannert e Hoyer em 1998 (More *et al*., 2016). Uma única injeção intracerebroventricular de 1 ou 3 mg/kg de STZ em ratos mostrou causar neuroinflamação crônica, a dilatação dos ventrículos e atrofia do septo com redução das contagens de células neuronais. Ambas as concentrações de STZ causam estes efeitos; entretanto, eles são mais pronunciados em 3 mg/kg. Em suma, o modelo STZ icv não exibe apenas neuroinflamação, mas também reproduz patologias tau e amilóide, bem como déficits cognitivos semelhantes a DA com uma cronologia compatível com a hipótese de inflamação da doença (NAZEM *et al*., 2015).

A injeção de estreptozotocina nos ventrículos laterais do rato produz distúrbios metabólicos, neuropatológicos e comportamentais crônicos remanescentes da doença de Alzheimer humana, mas falta uma explicação mecanicista aceitável para esses fenômenos (GRIEB, 2015).

1.3 O PAPEL DA NEUROINFLAMAÇÃO NA DOENÇA DE ALZHEIMER

Micróglia são as células imunes residentes do cérebro, e acredita-se serem as principais células responsáveis por iniciar a resposta imune à patologia da DA (HOPPERTON et al., 2017).

A neuroinflamação, manifestada pela ativação microglial, é um componente importante da patologia da doença de Alzheimer, com evidências sugerindo que é tanto uma reação ao processo da doença, como um contribuinte para danos neuronais (ZOTOVA et al., 2013), os sinais de ativação microglial na doença, avaliados por ampla análise morfológica, foram descritos pela primeira vez por Alois Alzheimer em 1906. Desde então, muitos grupos demonstraram claramente a estreita relação entre as placas Aβ e a micróglia ativada tanto em pacientes com DA como em modelos de camundongos (MCQUADE et al., 2019).

Embora a ativação precoce da micróglia seja benéfica para remover Aβ tóxica do cérebro, com o tempo a estimulação crônica da micróglia por Aβ também pode ser deletéria e levar à inflamação prolongada, deposição excessiva de Aβ e aceleração do processo neurodegenerativo. Durante a patogênese da DA, a atividade fagocitária da micróglia parece diminuir, enquanto a produção de citocinas pró-inflamatórias e moléculas neurotóxicas se agrava (WANG et al, 2019).

Descobertas recentes revelaram que a micróglia pode modular o processo de patogênese da DA interagindo ativamente com neurônios, astrócitos e oligodendrócitos. Um subtipo funcional recentemente caracterizado de astrócitos reativos, chamados astrócitos A1, mostrou acelerar e exacerbar a morte de neurônios e oligodendrócitos. A micróglia classicamente ativada induz astrócitos funcionalmente alterados através da liberação de IL-1α, TNF-α e C1q. Esses astrócitos A1 são incapazes de promover a sobrevivência neuronal, o crescimento, a sinaptogênese e a fagocitose. Em resumo, a micróglia pode ter efeitos benéficos ou prejudiciais durante o início e a progressão da DA, dependendo se o resultado predominante é a depuração de amilóide Aβ ou a liberação de mediadores inflamatórios (WANG et al, 2019).

Allograft Inflammatory Factor-1 (*AIF1*), também conhecido como gene que codifica a proteína adaptadora de ligação ionizada de cálcio (Iba1), que é uma proteína citoplasmática altamente expressa na linhagem monocítica, incluindo macrófagos e micróglia, tem sua expressão aumentada no processo inflamatório (KISHIKAWA et al., 2017). Elevações em marcadores neuroinflamatórios são amplamente relatadas na doença de Alzheimer (DA), tanto em pacientes humanos quanto em modelos animais (HOPPERTON et al., 2017).

A glicogênio sintase quinase-3 B (gene *GSK3B*) é a principal tau fosforilante da quinase e uma das quinases sugeridas que está envolvida na sua hiperfosforilação aberrante na DA. Em pacientes com DA, foi detectada expressão sistêmica aumentada de *GSK3B* ativa. A atividade da *GSK3B* perturbada está ligada a eventos relacionados à idade, como comprometimento da memória, produção de β-amilóide e neuroinflamação. Isso implica que a *GSK3B* desempenha um papel central na patogênese da DA e pode estar diretamente envolvida na perda de neurônios observada na DA (KETTUNEN et al., 2015).

2 OBJETIVOS

2.1 OBJETIVO GERAL

Investigar alterações na expressão dos genes *AIF1* e *GSK3B* no modelo animal da doença de Alzheimer esporádica em ratos lesados com STZ-ICV.

2.2 OBJETIVOS ESPECÍFICOS

- Avaliar os efeitos da injeção ICV de STZ sobre a memória espacial de curta duração nos testes de reconhecimento de objetos e labirinto em Y;
- Avaliar locomoção e atividade exploratória dos animais no teste de campo aberto;
- Avaliar variação de peso dos animais durante o experimento;
- Avaliar a expressão dos genes *AIF1* e *GSK3B* no modelo STZ ICV

3 ARTIGO CIENTÍFICO

Os materiais e métodos, resultados e discussão do trabalho encontram-se no artigo científico a seguir.

Gene expression in the animal model of Alzheimer's disease induced by streptozotocin

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Abstract: Alzheimer's disease (AD) is the most frequent form of dementia, it is a neurodegenerative disorder that worsens over time. Although the causes are still not fully understood, it is known that the disease is associated with the accumulation of plaques in the extracellular space, called senile plaques and neurofibrillary tangles in the intracellular environment of the neurons, causing neuronal death and loss of synapses, favoring the progressive decline. Elevations in neuroinflammatory markers are widely reported in the brains of AD patients and also in animal models. The animal model of Alzheimer's disease resulting from the intracerebroventricular (icv) infusion of streptozotocin (STZ) shows many aspects of sporadic AD, however there is no study thus far demonstrating the expression of *AIF1* and *GSK3B* genes in rat brain in the STZ-ICV induced model of sporadic Alzheimer's disease. Thus, the objective of this study was to investigate alterations in the expression of the *AIF1* and *GSK3B* genes in the animals infused with STZ-ICV. The animals were injected with STZ-ICV or vehicle and were evaluated in behavioral tests in the period of four weeks, on day 30 they were euthanized and the brains dissected for quantification of the gene expression of the *AIF1* and *GSK3B* genes in the hippocampus and prefrontal cortex. Thirty days after surgery, STZ-infused animals exhibited short-term-spatial memory (Y maze) and short-term recognition memory in the object recognition test (ORT) impairments. *AIF1*, but not *GSK3B*, gene expression was significantly elevated only in the hippocampus of the STZ-ICV group compared to the Sham group. These data suggest a participation of neuroinflammation in the cognitive decline observed in this animal model of sporadic AD.

Keywords: Sporadic AD, recognition memory, spatial memory, neuroinflammation, *AIF1*, *GSK3B*.

1. Introduction

Alzheimer's disease (AD), the most frequent form of dementia, is a progressive, continuous neurodegenerative disorder that affects large areas of the cerebral cortex and hippocampus (BLENNOW et al., 2006; MASTERS et al., 2015), it is the most prevalent neurodegenerative disorder in the world, affecting about 24 million people and it is estimated that by 2050 this number will be quadrupled, estimates in 2015 showed that about 46.8 million people are affected by dementia worldwide (PICANÇO et al., 2018).

Depending on the age of onset, AD is classified in two types: early onset or familial AD and late onset or sporadic AD. Familial AD is associated with mutations in the genes of presenilin and amyloid precursor protein (APP), whereas sporadic AD is mainly associated with the constant weakening and damage of nerve cells and brain function with aging (DUBEY et al., 2019). Most cases of AD are sporadic and result from multiple etiological factors, including environmental, genetic and metabolic factors. Alzheimer's disease is associated with accumulation of insoluble β-amyloid (Aβ) protein in plaques in the extracellular space, as well as in the wall of blood vessels, and aggregation of microtubule tau protein in neurofibrillary tangles in the intracellular environment of neurons (MASTERS et al., 2015), causing neuronal death and loss of synapses, all of which contribute to progressive cognitive decline (KOCAHAN, et al., 2017).

The animal model generated by the intracerebroventricular (ICV) administration of streptozocin (STZ), shows many aspects of sporadic AD (CHEN et al., 2013) as cognitive and cholinergic deficits of the brain, oxidative stress as well as a decrease in cerebral glucose / energy metabolism and insulin-resistant brain state (SALKOVIC-PETRISIC et al., 2013).

Chemically, STZ is a glucosamine - nitrosourea obtained from a soil microorganism *Streptomyces achromogenes* and has been extensively investigated for its potential to induce diabetes in animals. The animal model of STZ-ICV was introduced by Lannert and Hoyer in 1998 (MORE et al., 2016). A single ICV injection of 1 or 3 mg/kg of STZ in rats showed to cause chronic neuroinflammation, dilation of the ventricles and septal atrophy with reduction of neuronal cell counts. Both concentrations of STZ cause these effects; however, they are more pronounced at 3 mg/kg. Moreover, the STZ icv model does not only exhibit neuroinflammation but also reproduces tau and amyloid pathologies as well as cognitive deficits similar to AD with a chronology compatible with the disease inflammation hypothesis (NAZEM et al., 2015).

Neuroinflammation, manifested by microglial activation, is an important component of the pathology of Alzheimer's disease, with evidence suggesting that it is both a reaction to the disease process and a contributor to neuronal damage (ZOTOVA et al., 2013). Microglia are the resident immune cells of the brain, and are believed to be the major cells responsible for initiating the immune response to the pathology of AD (HOPPERTON et al., 2017). Allograft Inflammatory Factor-1 (*AIF1*), also known as a gene encoding the ionized calcium binding adapter protein (Iba1), which is a highly expressed cytoplasmic protein in the monocytic lineage, including macrophages and microglia, and has its expression increased in inflammatory process (KISHIKAWA et al., 2017). Elevations in neuroinflammatory markers are widely reported in Alzheimer's disease (AD), both in human patients and in animals models (HOPPERTON et al., 2017).

Glycogen synthase kinase-3 beta (gene *GSK3B*) is the main kinase phosphorylating tau and one of the suggested kinases is involved in aberrant hyperphosphorylation in AD. In patients with AD, increased systemic expression of active *GSK3B* was detected. Disturbed *GSK3B* activity is linked to age-related events, such as memory impairment, β-amyloid production and neuroinflammation. This implies that *GSK3B* plays a central role in the pathogenesis of AD and may be directly involved in the loss of neurons observed in AD (KETTUNEN et al., 2015).

To our knowledge, there is no study thus far demonstrating the expression of the *AIF1* and *GSK3B* genes in rat brain in the STZ-ICV-induced sporadic DA model. Thus, the objective of this study was to investigate alterations in the expression of the *AIF1* and *GSK3B* genes in the animals infused with STZ-ICV.

2. Methods

2.1 Animals

Were used male Wistar rats from our breeding colony, 3-4 months old and weighing 280-310 g at the beginning of the experiment. The animals were randomly housed in groups of four to five in polypropylene cages and maintained in a temperature-controlled room (22±2 °C) on a 12 h light-dark cycle (lights on at 7:00 a.m.). Before beginning any experimentation, the rats were allowed to acclimate to then environment and handling for at least 15 days. The animals had free access to food and water throughout the experiment. The experimental protocol complied with the recommendations of the Federal University of Parana and was approved by the University Ethics Committee (CEUA protocol 1089/2017).

2.2. Drugs

Streptozotocin (STZ) was purchased from Santa Cruz Biotechnology Inc., Santa Cruz, CA.

2.3. Experimental design

The rats were randomly distributed into two groups (n = 8 to 11 per group): sham group received only 0.9% sterile saline and STZ injured group (3 mg/kg, dissolved in sterile saline solution), both groups received injection of 4.5 µl per injection side into the lateral ventricles. The animals were weighed at the beginning of the experiment and every 7 days after surgery, until the 28th day. The animals were evaluated in behavioral tests, on day 27 passed by the Object Recognition Test (ORT), on day 28 in the Y maze spatial version for short-term spatial memory evaluation and open field test (OFT) and at day 30 were euthanized and the brains were dissected for quantification of the gene expression of the *AIF1* and *GSK3B* gene in the hippocampus and prefrontal cortex. (Fig. 1)

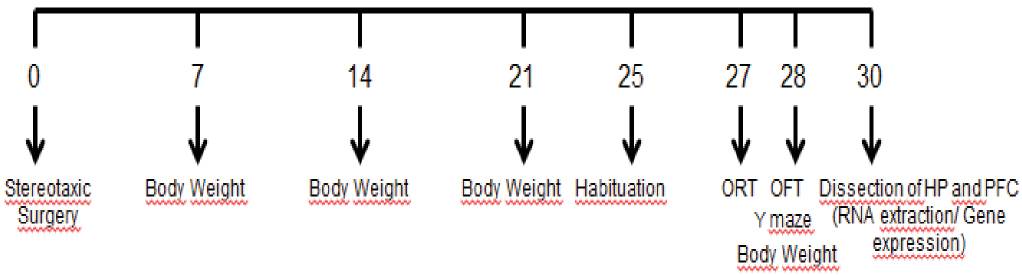

Figure 1. Experimental design. One set of animals was used in this study. The experimental design was conducted to evaluate the quantifying the gene expression of the *AIF1* and *GSK3B* gene and was performed to evaluate the late effects caused by STZ-icv on behavior. Hippocampus (HP); prefrontal cortex (PFC); open field test (OFT); object recognition test (ORT).

2.4 Stereotaxic surgery

For bilateral icv infusion of STZ, the animals were initially anesthetized with equitesin (distilled water, chloral hydrate, magnesium sulfate, 90% alcohol, thiopental and propylene glycol) 0.3mg/kg. Thereafter, they received atropine sulfate (0.4 mg/kg, i.p.). Soon after, they were positioned in a stereotaxic (David Kopf, model 957L) and then using a scalpel the skull was exposed. The following stereotactic coordinates were adjusted for the lateral ventricles: anteroposterior -0.8 mm, lateral ± 1.5 mm and dorsal-ventral -4.0 mm relative to bregma, according to Paxinos and Watson (1986). Through a hole in the skull, the micro infusion was performed with the aid of a needle (30 gauge) connected to a polyethylene tube adapted to a 10 μl micro-syringe (Hamilton, USA) that was attached to an infusion pump (Havard Apparatus, USA). The injured group received bilateral STZ injection (3 mg / kg body weight dissolved in sterile saline solution, 4.5 μl per injection site, total 9 μl). The sham group underwent the same surgical procedure, but the same volume of sterile saline solution was injected instead of STZ. After the surgical procedure, the animals were placed in individual boxes with a warm environment to recover the anesthesia and then sent to the maintenance room. All rats received Pentabiotic® (0.1 ml, intramuscular) to prevent infections. Water and food were placed inside the box 1 to 15 days after surgery so that the animals had no difficulty feeding themselves and to avoid trauma, in the following days they were arranged in a habitual way on the grid.

2.5. Object Recognition Test (ORT)

Object recognition test was performed to evaluate short-term recognition memory, performed between days 25 and 27 after surgery. In order to carry out both tests, a wooden box was used with the following measures: 100 x 100 x 40 cm, made of wood, painted black and positioned in a moderate light environment (20 lux). The evaluation of the behavior of the animals was made later through a camera positioned over the arena, and the animals behavior was recorded for later evaluation. (BASSANI et al., 2017a).

The animals were first habituated to the arena, placing each animal in the empty box for 5 min for free exploration. After 24 hours a new session of habituation of 5 min. After 1 h of interval, the training session was performed, positioning 2 identical objects in a symmetrical way in the apparatus, with a distance of the walls of around 10 cm. The animals were able to explore the objects for 5 min and then placed in their usual box. After 24 hours a new training session was performed with 2 other identical objects arranged in a symmetrical position, with a distance of the box walls of approximately 10 cm. After 1 h interval, each rat was again placed in the arena for the 3 min test session. One of the objects called a familiar object was kept in the training session and the other was replaced considered the new object and both remained in the original positions (DE BRUIN et al., 2011).

The materials of the objects were plastic, glass or ceramic. To avoid interference by olfactory stimuli the objects and box were cleaned with 10% alcohol and dried before each session and between each animal. Objects were randomly arranged to decrease a possible preference for specific objects. The objects were heavy enough that an animal could not move it. All animals were placed in the arena always facing the same wall (DE BRUIN et al., 2011). The Exploration of Objects was defined as the animal sniffing or touching the objects with the muzzle and was counted using manual stopwatches. It was not considered exploratory behavior to sit or walk around objects. (MELLO-CARPES; IZQUIERDO, 2013).

The basic measures performed for both tests were the total time spent by each animal exploring each object during the test session. The time spent by the animal exploring the familiar object (ORT) and the new object (ORT) are represented by 'a' and 'b', respectively. The following variables were calculated: $e = a + b$; and $d = (b - a) / e$. The variable 'e' is the sum of the total time of exploration of both objects during the test session; and 'd' is considered an index of discrimination between the new and the familiar (objects). In addition, 'd' is a relative measure of discrimination that considers the exploratory activity of the animal (e); (DE BRUIN et al., 2011).

2.6 Y maze spatial version

In this test it was used a device in the form of a 'Y', made in wood and painted black, which has 3 arms separated by angles of 120º, with the following measures, 50 cm long, 12 cm wide and 27 cm of height. The apparatus was placed in a moderate light environment (20 lux) and the test was filmed with a camera fixed to the ceiling and the behavior of the animals was evaluated later. (BASSANI et al., 2017b).

The spatial version of this test was performed in two parts being a training session and a test session with 1 h interval between the two. In the training session, one of the arms was inaccessible by a wooden door placed in front of him. Each animal was placed in one of the other 2 arms (called the 'initial arm', which was random between groups) that was free to explore these arms for 5 min, after that time the animal was removed and returned to its box habitual. The wooden door was removed, allowing access to the 3 arms of the maze; the previously inaccessible arm was called the "new arm". After the interval of 1 h, in the test session, each animal was placed again in the respective initial arm and was free to explore for 3 min. The short-term spatial memory was evaluated according to the time spent by each animal exploring the new maze arm, which should be significantly higher than 33.3% of the total, corrected for the latency to leave the initial arm and the time spent in the center of the appliance. Entry into an arm was considered when all the animal's paws were inside the arm. The Y maze apparatus was cleaned with 10% alcohol and dried between each session and each animal to prevent interference. (SIERKSMA et al., 2013).

2.7 Open field test (OFT)

This test was used to determine motor alterations. The apparatus consisted of a circular arena (97 cm diameter, 42 cm wall height) painted in white, divided with black lines into 19 quadrants and 3 concentric circles. The animals were placed singly in the center of the open field and allowed to freely explore the area for 5 min. Two motor parameters were assessed through this test: locomotion frequency (i.e., the number of crossings from one quadrant to another, was considered when all the animal's paws were inside the quadrant.) and rearing frequency (i.e., the number of times the animals stood on their hind paws) (BASSANI et al., 2014). Experiment the open field apparatus was washed with a 10% water-alcohol solution to eliminate possible odors left by other animals.

2.8 Real-time Polymerase Chain Reaction (RT-PCR)

All animals were euthanized after the last behavioral test. Total RNA was extracted from the hippocampus and frontal cortex of rats using mirVana ™ PARIS ™ Kit (Life Technologies), RNA was treated with DNase I – RNAse-free (Thermo Scienttific) and reverse-transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. Real-time PCR was conducted using TaqMan probes for *AIF1* and *GSK3B* genes and the gene expression levels of genes was normalized to the gene expression levels of all genes were normalized to *GAPDH* and *ACTB*.

2.9 Statistical Analysis

Two-way ANOVA followed by post hoc Bonferroni test were performed to analyze body weight data. Two-tailed Student's *t*-test for independent samples was performed to evaluate the open field test, object recognition test and Y maze spatial version. The statistical analyses were performed using GraphPad Prism, version 5.0. The error bars are reported as mean ± SEM of the mean. Significance level was set at $P < 0.05$.

3. Results

3.1 Behavioural Studies

3.1.1 Object Recognition Test (ORT) and Y Maze spatial version

In the ORT, there was a decrease in the discrimination index (Fig 2a, P = 0,007) in the STZ group in comparison to sham indicating short-term recognition memory impairment in the lesioned group STZ icv, as well as the Y maze test in the spatial version (Fig. 2b, P = 0.0006) in which the injured animals showed a decrease in the time spent by each animal exploring the new maze arm showing short-term spatial memory impairment.

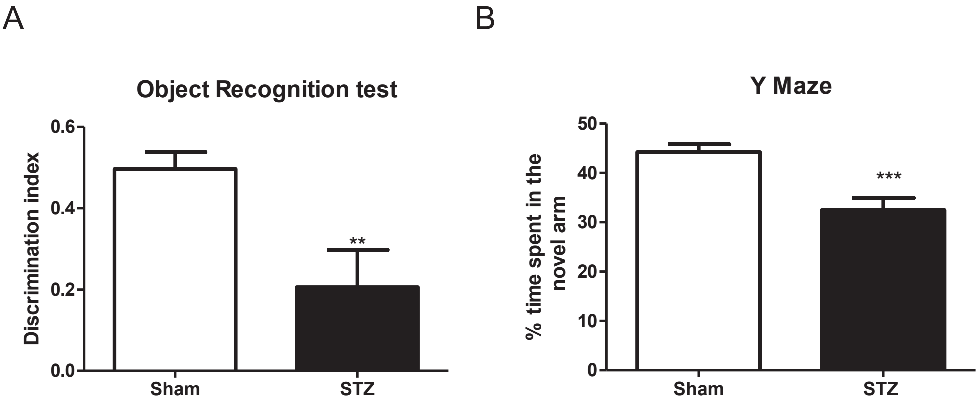

Figure 2. Behavioral evaluation of STZ-lesioned animals compared to sham rats. Cognitive performance was evaluated in the Object Recognition Test (A) and Y maze spatial version (B). Data are reported as mean ± SEM. $^{**}p<0.01$; $^{***}p<0.001$ vs. Sham group. Two-tailed Student's *t*-test for independent samples. (n = 10-11/group)

3.1.2 Open Field Test

The frequency of locomotion (total number of crosses, Fig. 3a, P = 0.066) and the rearing frequency (Fig. 3b, P = 0.281) showed no significant difference between the streptozotocin injected group compared to the sham group.

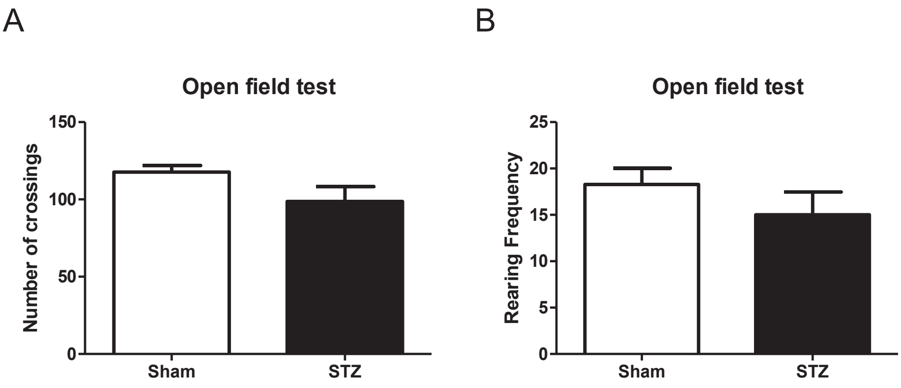

Figure 3. Behavioral evaluation of STZ-lesioned animals compared to sham animals. Locomotion and rearing frequencies were assessed in the open field test (A and B). Two-tailed Student's *t*-test for independent samples. (n = 8-11/group)

3.2 Effects of STZ-ICV on *AIF1* and *GSK3B* gene expression in the prefrontal cortex and hippocampus.

The STZ icv group showed no significant change in the expression of the *AIF1* gene in the prefrontal cortex (Fig. 4a, P = 0.91) when compared to the sham group. However, in the hippocampus (Fig. 4b, P = 0.04), the gene ratio was shown to be significantly elevated in the STZ icv group compared with the sham group.

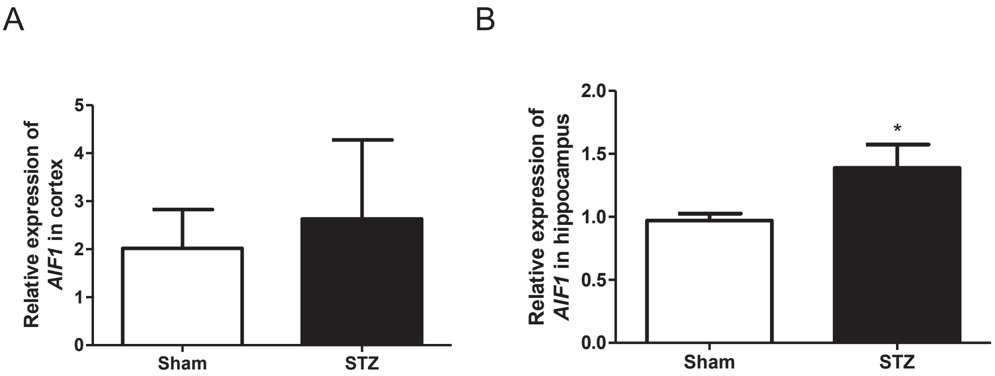

Figure 4. Expression of *AIF1* gene in the prefrontal cortex (A) and hippocampus (B) in STZ and SHAM rats as determined by RT-qPCR. *AIF1* = allograft inflammatory factor 1. Data are reported as mean ± SEM. * $p < 0.05$ vs. group Sham. Two-tailed Student's t test for independent samples. (n = 10-11/group)

Expression of the *GSK3B* gene in the prefrontal cortex (Fig. 5a, P = 0.75) and hippocampus (Fig. 5b, P = 0.06) showed no significant difference in the STZ icv group compared to the sham group.

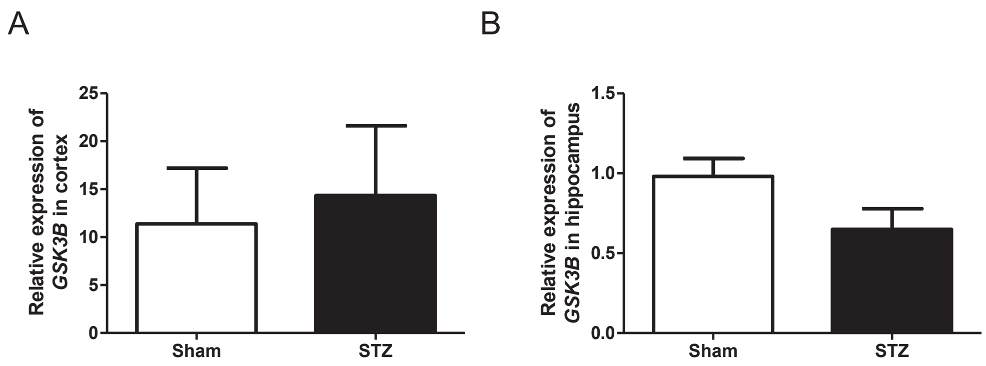

Figure 5. Expression of *GSK3B* gene in the prefrontal cortex (A) and hippocampus (B) in STZ and SHAM rats as determined by RT-qPCR. *GSK3B* = Glycogen synthase kinase-3 beta. Two-tailed Student's t test for independent samples. (n = 10-11/group)

3.3 Body Weight Measure

Body weight was significantly reduced in the STZ icv group from day 7 to day 28 when compared to sham (P<0.01, P<0.001, Fig 6).

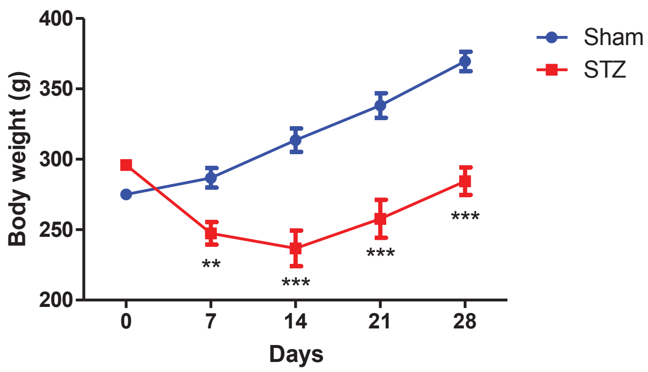

Figure 6. Changes in body weight. A) Icv-STZ induced a decline in body weight that lasted for about two weeks. Data are reported as mean ± SEM. ** p <0.01; *** p <0.001 vs. group Sham. (Two-way ANOVA followed by Bonferroni post-hoc test). (n = 10-11/group).

4. Discussion

The present study sought to investigate the alterations in the expression of the *AIF1* gene (encoding the Iba-1 protein) and *GSK3B* in rats infused with STZ-ICV.

Dementia is a brain disorder that causes progressive changes in cognitive functions, such as memory, language, rational thinking, and social skills, as well as emotion and personality (CIPRIANI et al., 2015). The main clinical phenotype of patients with AD is the progressive decline of cognition. Therefore, any valid animal model of AD should show cognitive impairment (CHEN et al., 2013). Streptozotocin (STZ) icv is a model of memory impairment well established in rodents and used to study the pathology of AD (RAI et al., 2014; BASSANI et al., 2017b).

The object recognition test is based on the known object scan difference as opposed to an object other than the habituation test. (DE BRUIN et al., 2011). Regarding ORT, performed on day 27 after surgery, animals injected with streptozotocin exhibited deficiencies in short-term recognition memory evidenced by the significant difference between the mean time spent exploring the new and familiar object. Many studies have shown cognitive deficits in animals injected with STZ and the results of this work are in agreement with other studies and publications (VILLAR et al., 2018; KHERADMAND et al., 2018; BASSANI et al., 2017a; ROSTAMI et al., 2017).

The Y maze test spatial version served as a measure for short-term spatial memory evaluation and the results suggest that the spatial memory of the STZ-icv group rats was impaired because the percentage of time spent on the novel arm was significantly lower than that of the sham group. This behavior has been extensively reported in animals receiving STZ icv (BASSANI et al., 2017b; SIERKSMA et al., 2013).

The open field test was performed to evaluate the locomotion and the exploratory activity of the animals, there was no difference in the locomotion and rearing frequency between the STZ icv and sham groups, as well as results demonstrated in other studies (BLOCH et al., 2017; ROSTAMI et al., 2017). The results confirm that there was no impairment in the locomotor activity, thus demonstrating that there were no motor impairments that could be related to the performance of the rats in the other cognitive tests, such as ORT and the Y Maze spatial version.

The animals body weight change was evaluated during the experiment, observing significant changes of the rats injected with STZ icv. In the first and second week, most infused rats lost 16 to 20% of their initial weight, this weight loss in this animal model of dementia is in agreement with changes already demonstrated in other studies (VILLAR et al., 2018; BLOCH et al., 2017).

There is a close association of neuroinflammation with the pathogenesis of AD that involves the activation of glial cells in neurodegenerative diseases. However, these normal glial functions can sometimes result in a more severe and chronic neuroinflammatory cycle that actually promotes neurodegenerative diseases (KAMAT et al., 2015). A previous study showed that in this animal model of sporadic AD there is a persistent and more pronounced microglial reaction in the CA3 area of the dorsal hippocampus, in which the total number of positive cells and Iba-1 reactive cells increased in this region 30 days after injection of STZ icv (BASSANI et al., 2017a). According to this result the present work using the RT-qPCR method which has been widely used for gene expression analysis due to its specificity, sensitivity and precision (PARK et al., 2013) showed that in the hippocampus (Fig. 4b, P = 0.04) the expression of the *AIF1* gene (which encodes the Iba-1 protein) considered a reliable marker of microglia (WILHELMSSON et al., 2017) was significantly elevated in the STZ icv group compared to Sham.

GSK3β phosphorylates tau and probably contributes to tau hyperphosphorylation in neurofibrillary tangles. These findings suggest that GSK3β plays a central role in the pathophysiology of AD and has led many researchers to study the effects of GSK3β on cognition in rodent models of the disease and in patients with AD. A variety of molecular and pharmacological approaches suggest that increased GSK3β activity probably contributes to pathologies and cognitive impairment in AD rodent models (KING et al., 2014). The results obtained in this work related to expression of the *GSK3B* gene did not present a significant difference of the STZ icv group compared to the sham group, 30 days after surgery, suggesting that in this period the cognitive impairment is not related to *GSK3B* gene expression.

In conclusion, the present study demonstrated that the intracerebroventricular administration of streptozotocin was able to cause cognitive impairment, as shown in the object recognition and Y maze tests in the spatial version, in agreement with previously published data for this animal model. These data suggest that increased microglial reactivity observed in previous studies may be related to the induction of *AIF1* gene expression in the hippocampus of STZ-ICV-infused rats. These data corroborate previous work that suggests a participation of neuroinflammation observed in this animal model.

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4 CONCLUSÃO

Em conclusão, o presente estudo demonstrou que a administração intracerebroventricular de estreptozotocina foi capaz de causar comprometimento cognitivo, conforme demonstrado nos testes de reconhecimento de objeto e labirinto de Y na versão espacial, de acordo com dados previamente publicados para este modelo animal. Esses dados sugerem que o aumento da reatividade da micróglia observada em estudos anteriores pode estar relacionado à indução da expressão gênica do *AIF1* no hipocampo de ratos lesados por STZ-ICV. Esses dados corroboram trabalhos anteriores que sugerem uma participação de neuroinflamação observada nesse modelo animal. O aumento da expressão relativa do gene AIF1 no hipocampo, apesar de esperado devido ao aumento de Iba-1 já demonstrado nesse modelo leva a propor hipóteses para estudos posteriores que elucidem se há uma conexão entre o aumento do gene e o prejuízo cognitivo apresentado pelos animais.

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